Using Biacore to study Fc receptor function and its inhibition by small peptide ligands

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The issue of antibody recognition by Fc receptors remains critical in understanding antibody-mediated immune function. To this end, several crystal structures of the Fc portion of IgG and IgE in complex with their receptors have been determined (Garman et al., 2000; Radaev et al., 2001; Sondermann et al., 2000). These structures showed that both IgE and IgG bind to their respective receptors using residues located near the lower hinge region of their Fc portion. Two questions, however, were not resolved despite the resolution of the crystal structures.

Firstly, the contribution of the glycosylation of Asn_{297} on IgG to antibody-Fc receptor recognition was not clear. Despite the well-known conservation of this glycosylation site among antibodies and earlier evidence showing the importance of the carbohydrate to antibody function, no available crystal structures suggested a significant degree of contact between carbohydrate and Fc receptor. In the light of the disparity between the crystallographic and functional data, we decided to probe the role of the carbohydrate in antibody-receptor recognition using Biacore's SPR technology.

The second question was whether the antibody:Fc receptor interaction could be inhibited by small compounds designed for the treatment of autoantibody-mediated immune disorders. To address this issue, we used Biacore to evaluate several synthetic peptide inhibitors. This work was originally published in the Journal of Biological Chemistry (Radaev and Sun, 2001).

ASSESSING THE ROLE OF CARBOHYDRATE IN ANTIBODY RECOGNITION BY FC RECEPTORS

To investigate the contribution of glycosylation of Asn_{297} on IgG to binding Fc receptors, we designed a set of experiments in which the carbohydrate was removed enzymatically and binding to its cognate type III Fc receptor, FcγRIII, was compared to that of the native antibody. We chose Biacore firstly because of its high sensitivity and accuracy in defining low affinity interactions and secondly, for its ability to measure direct binding without using any enzyme-based readout system and thus avoiding complications in interpretation associated with other methods. Finally, the acquisition of data using Biacore is extremely rapid.

To be able to accurately compare results between different preparations of IgG, we chose to immobilize FcγRIII rather than the antibody on Sensor Chip CM5 using a standard amine coupling procedure. In this mode, the concentration and flow rate of the IgG preparations can be accurately controlled so that receptor binding levels and affinity (K_D) can be directly compared. The results showed that while native antibody and its isolated Fc domain bound to FcγRIII with a similar K_D of approximately 5 μM, their deglycosylated counterparts displayed a more than 10-fold reduction in affinity to K_Ds of 70 μM and >150

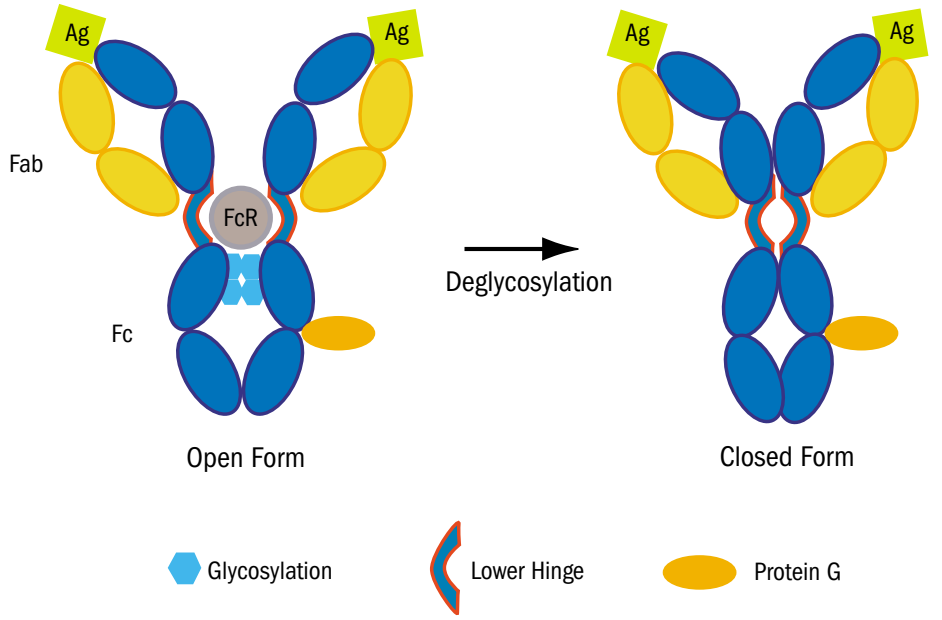

Figure 1. A model for the role of carbohydrate on Asn_{297} in IgG recognition by Fc receptors.

μM, respectively. This suggests that glycosylation of Asn_{297} plays an important role in receptor binding affinity.

To rule out the possibility that the reduction in affinity after deglycosylation was the result of denaturation or degradation of the antibody during the enzymatic deglycosylation treatment, the binding affinities of native Fc and deglycosylated Fc for protein G, were measured (Protein G recognizes the junction between the CH_2 and CH_3 domains rather than the lower hinge region of the antibody (see Figure 1)). In contrast to the drastic reduction in FcγRIII binding upon deglycosylation, removing the carbohydrates resulted in only a two-fold reduction in affinity for protein G, suggesting that the carbohydrates contribute specifically to FcγRIII binding and that their removal resulted in no global conformational changes.

To interpret these results in the context of crystallographic data that shows a lack of physical contact between the carbohydrates and the Fc receptor, we propose that the role of glycosylation on Asn_{297} of IgG is to stabilize the antibody conformation in an open form that favors receptor binding and that removal results in a change of Fc conformation to a closed form that is refractory to receptor binding (Figure 1).

MEASURING THE AFFINITY OF PEPTIDE:$F_c\gamma$RIII BINDING

Contact between the FcγRIII and IgG Fc occurs in the Fc lower hinge region. The ability of synthetic lower hinge peptides from various Ig molecules to bind to the receptor was tested using Biacore. The peptides were either immobilized on a sensor chip or were passed in solution over immobilized receptors; two experimental modes that produced distinctly different data quality.

The choice of ligand and analyte in designing Biacore experiments often depends on the availability or purity of the reagents, but the size of the interactants is also an important consideration. The detection signal at the chip surface is dependent on the masses of both interactants; a small analyte has less effect than a large analyte on the change in signal. In the case of small peptide:protein binding interactions, where the mass difference between the interactants can be large, the change in signal on binding will be greatly affected by the choice of which interactant to flow in solution. When FcγRIII was immobilized, peptide concentrations above 1 mM were necessary to detect any binding signal. In contrast, when peptides were immobilized, nanomolar concentrations of FcγRIII produced a signal. Using the latter experimental design, FcγRIII produced a signal at concentrations five-fold below the K_D value, enabling a wide range of concentrations to be tested in determining affinity.

Results from peptide immobilization experiments showed that peptides from IgG_1, IgG_2 and IgG_4 had affinities for FcγRIII of between 350 μM and 500 μM while peptides from IgE had an affinity of 1.3 mM. A disulfide-bonded dimer of IgG_1-derived peptides ($cIgG_1$) had a three-fold higher affinity for FcγRIII (K_D of 113 μM) than did monomeric peptides. There are two possible reasons for the increased affinity; increased avidity to the receptor or increased conformational stability of the peptide.

Although peptide immobilization produced more informative results than FcγRIII immobilization in our experiments, the optimal experi-

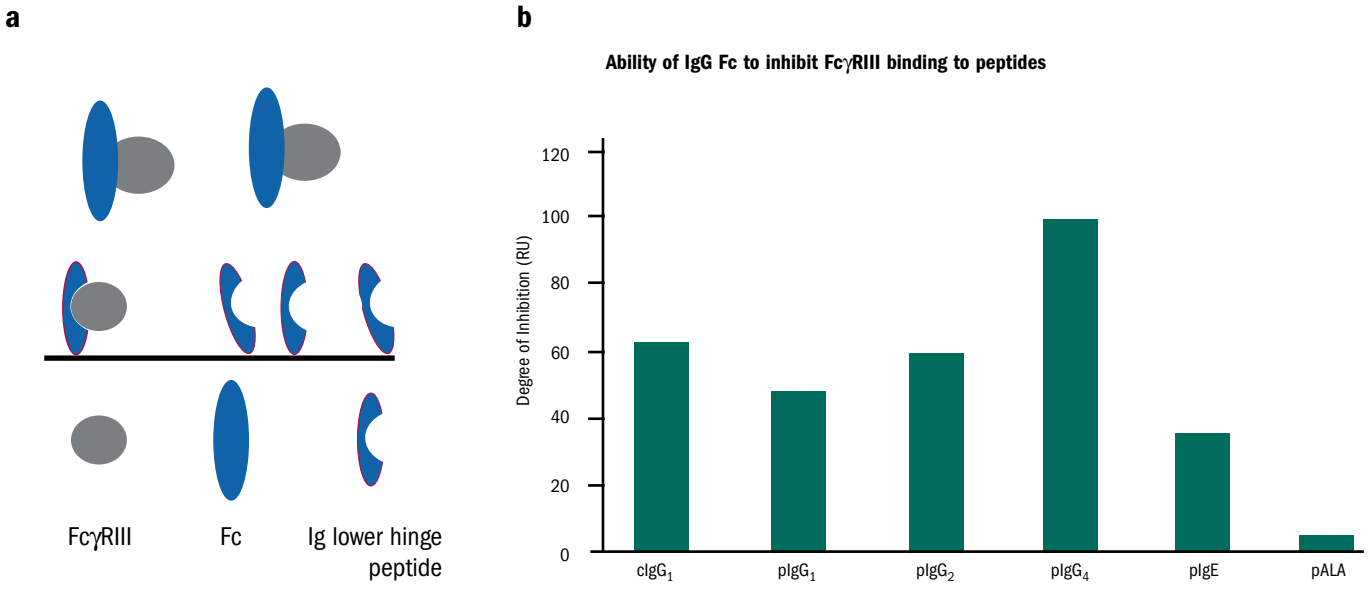

Figure 2. Peptide inhibition of Fc binding to FcγRIII. (a) experimental design: lower hinge peptides from various Ig types (IgG_1, IgG_2, IgG_4 and IgE) were immobilized on the surface of Sensor Chip CM5. FcγRIII was pre-incubated with Fc and passed in solution over the chip. (b) At a concentration of 10 µM Fc, there was little inhibition of FCγRIII binding to control peptide, pALA, and varying degrees of inhibition for other peptides.

mental design should be determined empirically as other factors, for example a lack of free amine groups on a prospective ligand, may rule out amine coupling.

TESTING THE ABILITY OF PEPTIDES TO COMPETE WITH IgFc FOR RECEPTOR BINDING

Inhibitors of Ig:Fc receptor binding interactions may have the potential to block the effects of autoimmune diseases since activation of Fc receptors are implicated in many autoimmune disorders including allergy and asthma. The Fc portion of autoantibodies binds to receptors on effector cells such as natural killer cells and macrophages, which may then be induced to release cytokines, leading to pathologic inflammation. The pattern of inflammation varies from disease to disease. For example, in rheumatoid arthritis and Chron's disease, inflammation remains localized while systemic lupus erythematosus is characterized by a systemic reaction. One strategy to inhibit Ig from binding to Fc receptors is to design peptides that mimic the receptor recognition site on Ig molecules. To this end, we designed several peptides representing the lower hinge region of IgG_1, IgG_2, IgG_4 and IgE.

To confirm that these synthetic peptides do compete with Fc binding to FcγRIII, two experimental strategies were designed to address two specific questions; firstly, does peptide-bound FcγRIII still permit binding to immobilized Fc? Secondly, does Fc-bound-FcγRIII still permit binding to immobilized peptide? Both strategies address the ability of the peptide to competitively inhibit Fc from binding FcγRIII. When we tested these alternative immobilization strategies, we found that Fc immobilization resulted in low receptor binding activity. Also, discernable inhibition with Fc immobilization required millimolar concentrations of peptide in solution. In contrast, peptide immobilization enabled the inhibition effect of Fc to be seen at micromolar concentrations.

With peptide immobilized on Sensor Chip CM5, FcγRIII was pre-incubated with increasing concentrations of Fc and passed over the chip (Figure 2a). An Fc concentration of 10 µM reduced binding of FcγRIII to the experimental peptides by 30-100 RU, compared to a 10 RU reduction when using a control peptide (Figure 2b). Although the Fc portion used in this experiment was from IgG_1, peptides from IgG_2 and IgG_4 also behaved equally as potent, or, in the case of IgG_4-derived peptides, superior competitive inhibitors, suggesting that antibody binding to Fc receptors is not restricted to one Ig sub-class.

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